2.0 METHODS FOR *IN VITRO* ER TA ASSAYS

2.1 Introduction

A number of different *in vitro* methods have been used to measure ER-induced transcriptional activation. However, currently, there are no generally accepted standardized methods for these assays. The *in vitro* assays used to identify ER agonists and antagonists can be classified into three broad groups: reporter gene assays using yeast cells; reporter gene assays using mammalian cells, and cell proliferation assays using mammalian cells. Most, but not all, of the mammalian cell lines used in the reporter gene assays lack an endogenous ER and must therefore be transiently or stably transfected with a plasmid carrying the ER and promoter genes. Yeast cells do not have an endogenous ER and must be transformed using an ER-containing plasmid. The cell lines used in cell proliferation assays contain an endogenous ER eliminating the need for transfection. The sensitivity and/or responsiveness of each cell line are determined by the characteristics of the cells themselves, the constructs used, and either by the efficiency of the transient transfection or by the concentration of the stably transfected/endogenous ER.

Many of the published *in vitro* ER TA studies were conducted specifically to test substances for ER agonist or antagonist activity; in addition, some studies have been conducted to investigate the process of ER-induced TA or to identify structure-activity relationships (SAR). Data from a total of 86 peer-reviewed publications and two submitted reports containing unpublished data have been extracted for this BRD. The publications and reports describe studies using a number of undefined genera of yeast (most likely *Saccharomyces cerevisiae*), various strains of *S. cerevisiae,* and a number of different mammalian cell lines. A list of the yeast strains and mammalian cell lines used in these studies are summarized in **Tables 2-1 to 2-3**. Information provided includes, where specified, the designation for the mammalian cell line or yeast strain, the ER source, the ER subtype, the investigators' designation(s) for the plasmid(s), the reference, and the designation for the assay used in this BRD. The BRD assay designation includes the name of the cell line or yeast strain, the source of the ER, the reporter gene product or endpoint assessed, and the product of any other plasmid. In addition, the tables indicate whether any vector used was stably or transiently transfected into the cell line or yeast.

Table 2-1 *In Vitro* Yeast ER TA Reporter Gene Studies (Arranged by Strain)

Species and Strain	Plasmids Transfected	BRD Designation	Reference
S. cerevisiae	hERα + ERE-LacZ	Yeast(*S.cer.*) hERα(S)+β-gal(S)	De Boever et al. (2001)
	hERα + PPK-ERE-LacZ	Yeast(*S.cer.*) hERα(S)+β-gal(S)	Elsby et al. (2001)
	hERα + ppK-vit2ERE-LacZ	Yeast(*S.cer.*) hERα(S)+β-gal(S)	Beresford et al. (2000); Miller et al. (2001)
	hER + ppK-vit2ERE-LacZ	Yeast(*S.cer.*) hER(S)+β-gal(S)	Routledge and Sumpter (1996; 1997); Harris et al. (1997); Odum et al. (1997); Moffat et al. (2001); Rajapakse et al. (2001); Yoshihara et al. (2001); Vinggaard et al. (1999; 2000);
S. cerevisiae 190	hERα + LacZ	Yeast(*S.cer.*190) hERα(S)+β-gal(S)	Morito et al. (2001a,b)
	hERβ + LacZ	Yeast(*S.cer.*190) hERβ(S)+β-gal(S)	Morito et al. (2001a)
S. cerevisiae 939	hER + ERE-CYC-1-LacZ	Yeast(*S.cer.*939) hER(S)+β-gal(S)	Chen et al. (1997)
*S. cerevisiae** 188R1	hER-CUP1 + A2ERE.cyc1.LacZ	Yeast(*S.cer.*188R1) hER(S)+β-gal(S)	Graumann et al. (1999)
S. cerevisiae BJ2168	mER + vitERE-LacZ	Yeast(*S.cer.*BJ2168) mER(S)+β-gal(S)	Ramamoorthy et al. (1997a)
S. cerevisiae BJ2407	hER + YRPE2-LacZ	Yeast(*S.cer.*BJ2407) hER(S)+β-gal(S)	Klotz et al. (1996)
	hER + vERE-CYC1-LacZ	Yeast(*S.cer.*BJ2407) hER(S)+β-gal(S)	Ramamoorthy et al. (1997a)
	hER + ERE-LacZ	Yeast(*S.cer.*BJ2407) hER(S)+β-gal(S)	Arnold et al. (1996)
S. cerevisiae BJ3505	hER + vitERE-LacZ	Yeast(*S.cer.*BJ3505) hER(S)+β-gal(S)	Ramamoorthy et al. (1997b)
	CUP1MET + 2FR.vit-iso1-cytC-LacZ	Yeast(*S.cer.*BJ3505) hER(S)+β-gal(S)	Coldham et al. (1997)
	hER-CUP1-MET + ERE-LacZ	Yeast(*S.cer.*BJ305) hER(S)+β-gal(S)	Gaido et al. (1997)
S. cerevisiae BJ-ECZ	hER + ERE2-CYC-1-LacZ	Yeast(*S.cer.*BJ-ECZ) hER(S)+β-gal(S)	Le Guevel and Pakdel (2001)
	rtER + ERE2-CYC1-LacZ	Yeast(*S.cer.*BJ-ECZ) rtER(S)+β-gal(S)	Petit et al. (1997, 1999); Le Guevel and Pakdel (2001)
*S. cerevisiae** CYT10-5d	hER + ERE-LacZ	Yeast(*S.cer.*CYT10-5d) hER(S)+β-gal(S)	Chen et al. (1997)

Species and Strain	Plasmids Transfected	BRD Designation	Reference
*S. cerevisiae** ER	hER + LacZ	Yeast(*S.cer*.ER) hER(S)+β-gal(S)	Tran et al. (1996)
*S. cerevisiae** ER179C	hER + LacZ	Yeast(*S.cer*.ER179C) hER(S)+β-gal(S)	Tran et al. (1996)
S. cerevisiae PL3	PL3-hERαdef + ERE-URA	Yeast(*S.cer*.-PL3) hERαdef(S)+β-gal(S)	Connor et al. (1996); Zacharewski et al. (1998)
S. cerevisiae YRG-2	hERα + ERE-CYC-1-LacZ	Yeast(*S.cer*.YRG-2) hERα(S)+β-gal(S)	Lascombe et al. (2000)

* Species name was not provided in publication, but is likely *S. cerevisiae.*
Abbreviations: β-gal = β-Galactosidase, ER = Estrogen receptor, ERE = Estrogen response element, h = Human, m = Mouse, r = Rat, rt = Rainbow trout, (S) = Stably transfected, (T) = Transiently transfected, vit = Vitellogenin.

Table 2-2 *In Vitro* Mammalian Cell ER TA Reporter Gene Studies (Arranged by Cell Line)

Cell Line	Plasmids Transfected	BRD Designation	Reference
BG-1	hER + ERE-MMTV-Luc	BG1 hER(E)+Luc(S)	Xenobiotic Detection Systems (2001)
	hER + MMTV-Luc	BG1 hER(E)+Luc(T)	Rogers and Denison (2000)
CHO-K1	hERα + ERE-hs-Luc	CHO-K1 hERα(S)+Luc(S)	Otsuka Pharmaceutical (2001)
	hERα + ERE-tk-Luc	CHO-K1 hERα(S)+Luc(T)	Otsuka Pharmaceutical (2001)
COS-1	mERα + vitA2EREB-Luc	COS-1 hERα(T)+Luc(T)	Tremblay et al. (1998)
	mERα + vitA2ERETK-Luc	COS-1 mERα(T)+Luc(T)	Tremblay et al. (1998)
	mERβ + vitA2EREB-Luc	COS-1 hERβ(T)+Luc(T)	Tremblay et al. (1998)
	mERβ + vitA2ERETK-Luc	COS-1 mERβ(T)+Luc(T)	Tremblay et al. (1998)
ELT-3	hER + vitERE-tk-Luc6a + CMV- LacZ	ELT-3 hER(T)+Luc(T)+β-gal(T)	Hodges et al. (2000)
HEC-1	hERα + C3-Ti-Luc	HEC-1 hERα(T)+Luc(T)+β-gal(T)	Sun et al. (1999)
	hERα + ERE-pS2-CAT	HEC-1 hERα(T)+CAT(T)+β-gal(T)	Meyers et al. (1999); Sun et al. (1999)
	hERα + ERE-pS2-CAT + CMVβ-gal	HEC-1 hERα (T)+CAT(T)+β-gal(T)	Kraichely et al. (2000)
	hERβ + C3-Ti-Luc	HEC-1 hERβ(T)+Luc(T)+β-gal(T)	Sun et al. (1999)
	hERβ + ERE-pS2-CAT	HEC-1 hERβ(T)+CAT(T)+β-gal(T)	Meyers et al. (1999); Sun et al. (1999)
	hERβ + ERE-pS2-CAT + CMVβ-gal	HEC-1 hERβ(T)+CAT(T)+β-gal(T)	Kraichely et al. (2000)
HEK293	hERα + 3ERE-tata-Luc	HEK293 hERα(S)+Luc(S)	Meerts et al. (2001)
	hERα + 3ERE-tata-Luc	HEK293 hERα(S)+Luc(T)	Seinen et al. (1999)
	hERα + 3ERE-tata-Luc	HEK293 hERα(T)+Luc(T)	Seinen et al. (1999)

Cell Line	Plasmids Transfected	BRD Designation	Reference
	hERα + ERE-Luc	HEK293 hERα(T)+Luc(T)	Collins-Burow et al. (2000)
	hERα + ERE-TATA-Luc + SV2-LacZ	HEK293 hERα(T)+Luc(T)+β-gal(T)	Kuiper et al. (1998)
	hERβ + 3ERE-tata-Luc	HEK293 hERβ(S)+Luc(S)	Meerts et al. (2001)
	hERβ + 3ERE-tata-Luc	HEK293 hERβ(S)+Luc(T)	Seinen et al. (1999)
	hERβ + 3ERE-tata-Luc	HEK293 hERβ(T)+Luc(T)	Seinen et al. (1999)
	hERβ + ERE-Luc	HEK293 hERβ(T)+Luc(T)	Collins-Burow et al. (2000)
	hERβ + ERE-TATA-Luc + SV2-LacZ	HEK293 hERβ(T)+Luc(T)+ β-gal(T)	Kuiper et al. (1998)
HeLa	GAL4HEG0 + 17m5-G-Luc	HeLa hERαdef(S)+Luc(S)	Connor et al. (1997); Moore et al. (1997); Zacharewski et al. (1998)
	hER + p17m5-G-Luc + AG60.neo	HeLa hER(S)+Luc(S)	Balaguer et al. (1996)
	hER + vitA2ERE-TK-CAT	HeLa hER(T)+CAT(T)	Miksicek (1993; 1994)
	hERα + ERE-Luc	HeLa hERα(T)+Luc(T)+β-gal(T)	Gaido et al. (1998)
	hERα + TK-XvitERE-Luc	HeLa hERα(T)+Luc(T)	Sumida et al. (2001)
	hERα + XvitERE-tk-Luc	HeLa hERα(T)+Luc(T)	Tarumi et al. (2000)
	hERβ + ERE-Luc	HeLa hERβ(T)+Luc(T)+β-gal(T)	Gaido et al. (1999)
	mER + ERE81CAT	HeLa mER(T)+CAT(T)	Makela et al. (1994); Shelby et al. (1996); Garner et al. (1999)
HepG2	hER + C3-Luc + β-gal	HepG2 hER(T)+Luc(T)+β-gal(T)	Ramamoorthy et al. (1997b)
	hERα + C3-Luc + CMV-β-gal	HepG2 hERα(T)+Luc(T)+β-gal(T)	Gaido et al. (1999; 2000)
	hERα + ERE-C3-Luc	HepG2 hERα(T)+Luc(T)+β-gal(T)	Gould et al. (1998)
	hERβ + C3-Luc + CMV-β-gal	HepG2 hERβ(T)+Luc(T)+β-gal(T)	Gaido et al. (1999, 2000)
	rERα + C3-Luc + CMV-β-gal	HepG2 rERα(T)+Luc(T)+β-gal(T)	Gaido et al. (1999)
	rERβ + C3-Luc + CMV-β-gal	HepG2 rERβ(T)+Luc(T)+β-gal(T)	Gaido et al. (1999)
Ishikawa	hER + vitERE-Luc + CMV-β-gal	Ishikawa hER(T)+Luc(T)+β-gal(T)	Klotz et al. (1996)
MDA-MB-231	hER + CKB-Luc	MDA-MB-231 hER(T)+Luc(T)	Ramamoorthy et al. (1997b)
	hERα + ERE-Luc	MDA-MB-231 hERα(T)+Luc(T)	Bonefeld-Jørgensen et al. (2001)
MCF-7	GAL4HEG0 + 17m5-G-Luc	MCF-7 hERαdef(T)+Luc(T)	Connor et al. (1996)
	GAL4HEG0 + 17m5-G-Luc + CH110 (β-gal)	MCF-7 hERαdef(T)+Luc(T)+β-gal(T)	Charles et al. (2000a,b)
	Gal4-HEG0 + 175m5-G-Luc + CMV-lacZ	MCF-7 hERαdef (T)+Luc(T)+β-gal(T)	Zacharewski et al. (1998); Fertuck et al. (2001a,b)
	GAL4HEG0 + 17m5-G-Luc + CMV (β-gal)	MCF-7 hERαdef (T)+Luc(T)+β-gal(T)	Clemons et al. (1998)
	GAL4-HEG0 + 17m5-G-Luc + lacZ	MCF-7 hERαdef (T)+Luc(T)+β-gal(T)	Fielden et al. (1997)
	GAL4-hERαdef + 17m5-G-Luc + CMV-lacZ	MCF-7 hERαdef(T)+Luc(T)+β-gal(T)	Matthews et al. (2001)
	GAL4-hERβdef + 17m5-G-Luc + CMV-lacZ	MCF-7 hERβdef(T)+Luc(T)+β-gal(T)	Matthews et al. (2001)

Cell Line	Plasmids Transfected	BRD Designation	Reference
	GAL4-mERβdef + 17m5-G-Luc + CMV-lacZ	MCF-7 mERβdef(T)+Luc(T)+β-gal(T)	Fertuck et al. (2001a,b)
	hER + CKB-CAT	MCF-7 hER(T)+CAT(T)	Ramamoorthy et al. (1997a,b)
	hER + ERE + Luc	MCF-7(MELN41)-hER(E)+Luc(S)	Lascombe et al. (2000)
	hER + (ERE)3-SV40-Luc + CMV	MCF-7 hER(E)+Luc(T)	Yoshihara et al. (2001)
	hER + ERE-tk-CAT + ON249(β-gal)	MCF-7 hER(E)+CAT(T)+β-gal(T)	Bonefeld-Jørgensen et al. (2001)
	hER + ERE2-Luc + CMV(β-gal)	MCF-7 hERαdef(E)+Luc(T)+β-gal(T)	Klotz et al. (1996)
	hER + ERE-tk-Luc + J7lacZ	MCF-7 hER(E)+Luc(T)+β-gal(T)	Jobling et al. (1995)
	hER + GV-tk-vEREx5-Luc	MCF-7 hER(E)+Luc(T)	Sumida et al. (2001)
	hER + Luc	MCF-7 hER(T)+Luc(T)	Ramamoorthy et al. (1997b)
	hER + Vit-CAT	MCF-7 hER(T)+CAT(T)	Connor et al. (1997)
	hER + Vit-tk-Luc	MCF-7 hER(E)+Luc(S)	Kramer et al. (1997)
	hER + vitERE2-Luc + CMV(β-gal)	MCF-7(M) hER(E)+Luc(T)+β-gal(T)	Collins-Burow et al. (2000)
T47D	hER + CAT	T47D hER(E)+CAT(T)	Nakagawa and Suzuki (2001)
	hER + EREtata-Luc	T47D hER(E)+Luc(S)	Legler et al. (1999); Meerts et al. (2001); Hoogenboom et al. (2001)

Abbreviations: β-gal = β-Galactosidase, CAT = Chloramphenicol acetyl transferase, (E) = Endogenous, ER = Estrogen receptor, ERE = Estrogen response element, h = Human, Luc = Luciferase, m = Mouse, r = Rat, (S) = Stably transfected; (T) = Transiently transfected, vit = Vitellogenin.

Table 2-3 *In Vitro* Mammalian Cell ER TA Cell Proliferation Studies (Arranged by Cell Line)

Cell Line	BRD Designation	Reference
Ishikawa	Ishikawa hER(E) + CP	Le Guevel and Pakdel (2001)
MCF-7	MCF-7 hER(E) + CP	Miksicek (1993); Makela et al. (1994); Soto et al. (1994); Soto et al. (1995); Dodge et al. (1996); Mellanen et al. (1996); Fielden et al. (1997); Harris et al. (1997); Ichikawa et al. (1997); Moore et al. (1997); Ramamoorthy et al. (1997a); Jones et al. (1998); Korner et al. (1998); Go et al. (1999); Miodini et al. (1999); Bonefeld-Jørgensen et al. (2001); Morito et al. (2001a); Nakagawa and Suzuki (2001); Otsuka Pharmaceutical (2001); Payne et al. (2001)
MCF-7 [focus assay]	MCF-7 hER(E) + CP(F)	Gierthy et al. (1997); Arcaro et al. (1998); Tamir et al. (2000)
MCF-7(Bos)	MCF-7(Bos) hER(E) + CP	Schlumpf et al. (2001)
MCF-7(BUS)	MCF-7(BUS) hER(E) + CP	Schafer et al. (1999)
MCF-7(E3)	MCF-7(E3) hER(E) + CP	Wiese et al. (1997); Vinggaard et al. (1999)
MCF-7(M)	MCF-7(M) hER(E) + CP	Collins-Burow et al. (2000)
T47D	T47D hER(E) + CP	Makela et al. (1994); Mellanen et al. (1996); Schafer et al. (1999); Tamir et al. (2000)
ZR-75	ZR-75 hER(E) + CP	Jobling et al. (1995); Harris et al. (1997)
ZR-75-1	ZR-75-1 hER(E) + CP	Schafer et al. (1999)

Abbreviations: CP = Cell proliferation; (E) = Endogenous; ER = Estrogen receptor, (F) = foci; h = Human.

The ER used in the majority of reported *in vitro* ER TA studies was human in origin; a small number of studies used the ER derived from mouse or rainbow trout. As discussed in the ER Binding BRD, two human ER proteins have been isolated. These two proteins, known as ERα and ERβ, are found in different proportions in various human and mammalian tissues, and have different capacities for binding substances of certain chemical classes, particularly

phytoestrogens (Kuiper at al., 1997). Both receptors have been used in *in vitro* ER TA assays. ER-induced transcriptional activation is measured in reporter gene assays by following the production of an enzyme whose synthesis and expression is controlled by an ERE. The reporter plasmid typically contains an ERE which controls the expression of a reporter gene, usually luciferase (*Luc*), chloramphenicol acetyl transferase (*CAT*) or, in yeast, β-galactosidase (β-*gal*). Since the sequence of the ERE is contained within the frog vitellogenin gene, this has frequently been used as the ERE source for these assays. Some *in vitro* ER TA assays use cells that have been stably transfected with the ER or with the ER and the reporter gene vectors.

Regardless of whether transient or stably transfected cells are used in the assays, test substances that enter the cells interact with the ER, which becomes activated by a change in its conformation. The activated ER then binds with soluble cell factors, and the resulting complex binds to the ER response elements on the reporter plasmid. This binding initiates the expression of the reporter gene and the production of its associated enzyme. An appropriate substrate in the incubation mixture is metabolized by the newly synthesized enzyme, resulting in the production of an easily detected product. The majority of *in vitro* ER TA studies using mammalian cell lines have used luciferase to assess transcriptional activation because the assay is more rapid, more sensitive, and easier to perform than CAT-based assays. Also, in contrast to the luciferase-based assay, CAT assays require a radiolabeled substrate (either chloramphenicol or acetyl-CoA). In an alternative approach, binding of an appropriate substance to the endogenous ER stimulates cell division in an estrogen responsive cell line.

The cellular level of the ER, which affects the sensitivity of the assay, is usually two to five fold higher in cell lines transiently transfected with the ER compared to cell lines with an endogenous or stably transfected ER. However, the major disadvantage of using transiently transfected cell lines is the uncertainty of the efficiency of transfection, and hence the repeatability of the assay. An approach to monitor the efficiency of transfection is based on the transfection of a plasmid carrying a gene (typically β-*gal*) that codes for a protein that is produced constitutively into the cell line. The level of this enzyme in transfected cells is used as a measure of the transfection efficiency.

Cytotoxicity can be a complicating factor in *in vitro* ER TA assays, particularly when antagonism is being assessed. The absence of or a decrease in the ER-induced TA response might be the result of cell toxicity rather than reflecting the ability of the test substance to interact with the ER. Cell toxicity can be corrected for by performing a parallel cytotoxicity experiment or by measuring the product of a constitutively active gene transfected into the cell on a separate plasmid. Some of the mammalian cell lines transfected with *luc* or *CAT* reporter constructs have also been transfected with a plasmid coding for β-*gal*. The synthesis of β-galactosidase is independent of a receptor-mediated effect, and a comparison of its level in treated versus control cells can be used as a measure of treatment-related cell toxicity.

In studies to measure agonism, the ER-containing cells are treated with a test substance, and the induction of luciferase, CAT, or β-galactosidase determined. A number of measures have been used to assess whether the test substance induces ER-dependent transcriptional activation. These measures include the relative enzyme activity, usually expressed as the amount of the test substance that elicits a specific response compared to the reference estrogen, the EC_{50} of the substance, or a qualitative assessment of "positive," "negative," or "weak." Relative potencies have been presented also. These are determined by dividing the concentration of a test substance producing a half maximal response by the concentration of 17β-estradiol producing an equivalent response. When cell proliferation was used as the endpoint, a qualitative response measure was usually provided, but in some cases an EC_{50} or a fold increase compared to the reference estrogen was determined. In some studies, the parameter measured is determined graphically; in others a more complicated procedure using regression analysis has been used. In publications in which numerical data were not provided, values for the EC_{50} or for a relative activity versus the reference estrogen were estimated, if possible. These estimated values are italicized in **Appendix D**.

In the studies conducted to determine antagonism, the cells were treated simultaneously with the test substance and the reference estrogen, and the ability of the test substance to inhibit reference estrogen-induced TA measured. Qualitative results were usually provided, but in a few cases a relative value was presented.

In addition to mammalian cells, various strains of *S. cerevisiae* and other undefined yeast strains (probably *S. cerevisiae*) have been used to assess the ability of substances to act as ER agonists or antagonists. Since yeast cells do not contain an ER, the DNA sequence for the ER is transfected into the cells (Routledge and Sumpter, 1996). Usually, both the ER and reporter gene plasmids have been transfected simultaneously into the cells, and clones harboring both plasmids selected for simultaneously. The production of β-galactosidase has been used as the measure of ER-induced TA. The most commonly used expression plasmid contains the ER and CUP1-MET promoter, while the reporter plasmid contains the ERE (frequently the vitellogenin A2 ERE) and the iso-1-cytochrome c (CYC1) promoter in a *LacZ* fusion vector. Expression of the reporter gene results in the formation of β-galactosidase, which is usually measured following lysis of the cells, although a few investigators have measured enzyme activity in the medium. β-Galactosidase metabolizes a chromogenic galactopyranoside to a chromogenic agent that can be measured spectrophotometrically. For the yeast assays, the activity of β-galactosidase versus the log of the concentration of the test substance is represented graphically. Fold induction of β-galactosidase has also been presented.

Because there are no "consensus" cell lines, vectors, or specific treatment protocols for *in vitro* ER TA studies, the following sections describe general protocols for agonism and antagonism studies using mammalian and yeast cells transfected with both an ER and a reporter gene, cells containing an endogenous ER that are transfected with a reporter gene, and mammalian cells that use growth as an endpoint.

2.2 *In Vitro* Mammalian Cell ER TA Reporter Gene Assays

2.2.1 Expression and Reporter Gene Constructs

Mammalian cells are generally transfected with two or three different types of plasmids. The expression plasmid contains the ER, which is constructed by ligating the cDNA of the ER gene into a eukaryotic expression vector that contains a promoter, the human growth hormone transcription termination and polyadenylation signals, the SV-40 origin of replication, and an antibiotic resistance gene for selection. A number of genes with different termination and polyadenylation signals have been used in the various expression constructs used for *in vitro* ER TA studies. The reporter plasmid contains the *Luc* gene regulated by an ERE from the frog or

fish vitellogenin gene, with a response element that is upstream of the promoter derived from a mammalian cell (e.g., the *tk* gene or the mouse mammary tumor virus long terminal repeat [MMTV]). Rather than *Luc*, a few investigators have measured ER-induced TA using the *CAT* gene that is regulated by the pS2 or *tk* promoter. A third plasmid, when used, contains the cytomegalovirus (CMV) promoter upstream of β-*gal*, which induces the transcription of β-galactosidase in the cell. The β-*gal* plasmid is used to monitor transfection efficacy and toxicity of the test substance.

Before 1996, investigators were unaware that two forms of hER existed; thus, publications before that date stated simply that hER was used. After that date, most investigators stated explicitly whether they transfected cells with hERα or hERβ. It is assumed for those publications in which it is not stated, that the hERα gene was used since this gene is the predominant form in most female reproductive organs containing hER.

2.2.2 Stably and Transiently Transfected Cell Lines

The majority of *in vitro* ER TA studies abstracted for this BRD used transiently transfected cells, despite the fact that a new batch of transfected cells must be produced for each new experiment. Transfection is performed by exposing the cells to both the ER and reporter gene plasmids in the presence of calcium phosphate, liopofectamine, or commercially available transfection reagents, or by electroporation. These substances and electroporation increase cell membrane permeability, allowing for the passive uptake of the plasmids by the cells. These foreign DNAs are typically rejected by the cell within three to seven days after transfection. In cells that harbor an endogenous or stably transfected ER, only the reporter gene construct and, if used, the construct to assess cytotoxicity, need to be transfected. Some investigators have used antibiotic selection to manipulate the cell lines so that both the expression and reporter plasmids are stable. These stably transfected cell lines do not require genetic manipulation or cell transfection skills before performing the assay.

2.2.3 *In Vitro* Mammalian Cell ER TA Assays with a Reporter Gene

The following section provides a generic example of how *in vitro* mammalian cell reporter gene ER TA assays are typically conducted.

Mammalian cells, at a confluency appropriate for the cell type, are seeded into culture dishes or wells of microtiter plates and cultured for 18-24 hours at 37°C. The cells are then transfected with the appropriate plasmids. After incubation from 4-24 hours at 37°C to express the ER, the medium is removed and the cells are treated with the test substance dissolved in culture medium, absolute ethanol, or dimethyl sulfoxide (DMSO). The cells are incubated for a further 24-48 hours at 37°C, after which time the medium is aspirated, the cells are washed with an appropriate buffer, and then lysed with the same buffer containing $MgCl_2$, Triton X 100, and dithiothreitol. After 15 minutes at room temperature, followed by centrifugation for a short time to sediment cell debris, an aliquot of the supernatant is removed for measurement of the induction of the reporter gene product.

For the induction of luciferase, adenosine triphosphate (ATP) and coenzyme A are added in glycylglycine buffer to the cell lysate in a microtiter plate. Luciferin is added to start the reaction and the fluorescence measured using a microtiter plate luminometer. The data are expressed in relative light units. For the induction of CAT, an aliquot of the lysed cells is incubated with chloramphenicol and acetyl coenzyme A, one of which is radiolabeled (Gorman et al., 1982), for 30 minutes at 37°C, and samples are removed at various time points. The reaction is stopped with ethyl acetate, which extracts the radiolabeled, acetylated chloramphenicol. The organic (ethyl acetate) phase is dried, redissolved in ethyl acetate, and spotted on silica gel plates. The radioactive acetylated product is separated from the parent chloramphenicol using thin layer chromatography. The radioactive spots are located by autoradiography of the plates for 18 hours, cut out, and counted in a scintillation counter. More recently, a commercially available ELISA kit has been used to measure CAT activity. When β-galactosidase is used as a measure of toxicity, it is measured using chlorophenol red-β-D-galactopyranoside (CRGP) as the substrate. Following hydrolysis of CRGP by β-galactosidase, the intensity of the colored product is measured at 570 nm after a 30-minute reaction period, using a spectrophotometer.

In agonism studies, the cells are treated with a test substance and the induction of the reporter gene product is used to measure the response. To assess relative potency, the maximal fold-increase induced by the test substance can be compared with that induced by the reference

estrogen. Alternatively, when dose-response data are generated, the EC_{50} for the test substance can be calculated and compared with that for the reference estrogen. The reference estrogen is included to demonstrate the adequacy of the test system and for an assessment of relative potency.

For antagonism studies, the cells are exposed simultaneously to the reference estrogen and the test substance, while control cells are exposed to the reference estrogen only. The difference in induction of the reporter gene product in the presence and absence of the test substance is used as a measure of antagonism.

2.3 Yeast Cell ER TA Reporter Gene Assays

2.3.1 Expression and Reporter Gene Constructs

For yeast ER TA assays, the expression plasmid containing the ER is under the control of the CUP1 metallothionein promoter. Copper must be added to the medium to initiate synthesis of the ER. The reporter plasmid contains palindromic ERE's inserted upstream of the promoter of the CYC1 gene linked to the *LacZ* gene that codes for β-galactosidase. Generally, hER has been used, but one publication reported on the use of the mER and three publications reported using the rtER. Four publications specifically stated that ERα and/or ERβ were transfected into the yeast cells.

2.3.2 Yeast Cell ER TA Assays with a Reporter Gene

The following section provides a generic example of how yeast cell ER TA reporter gene assays are typically conducted.

S. cerevisiae containing a stably transfected hER and a construct containing the β-*gal* reporter gene are grown overnight at 30°C in an orbital shaker in appropriate selective medium. The next day, an aliquot of the overnight culture is grown to mid-log phase, and then diluted to an OD of 0.03 at 600 nm. The diluted yeast suspension is aliquoted into a microtiter plate or small tubes and the test substance dissolved in ethanol or DMSO is added. Because hER in these cells is linked to the CUP1 promoter, 50 μM of copper sulfate ($CuSO_4$) is added to the yeast culture to induce receptor production. The cells are incubated overnight at 30°C with vigorous shaking and

the OD is read at 600 nm to assess cell growth or toxicity. A diluted aliquot of the cells is then pipetted into a microtiter plate. Assay buffer containing the color reagent, *o*-nitrophenylgalactoside (OPNG), and a lysing solution (containing sodium dodecyl sulfate [SDS], mercaptoethanol, and oxalyticase) is added to the cells. The increase in production of o-nitrophenol by the induced β-galactosidase is measured at 420 nm using a microtiter plate reader. The OD is also measured at 550 nm to correct for colorimetric distortion due to debris. β-Galactosidase activity is calculated according to the Miller equation where T = minutes of

$$\text{Miller Units } (A_{420}/\text{min/mL cells/OD}_{600}) = 1000 \times \frac{OD_{420} - (1.75 \times OD_{550})}{T \times V \times OD_{600}}$$

reaction time and V = volume of assay in mLs. Data are expressed in various formats, including absorbance at 550 nm versus the log of the molar concentration; relative potency versus the response with estrogen; β-*gal* activity (% of control versus log concentration); and fold induction of β-*gal* activity compared to induction by the reference estrogen.

In agonism studies, the yeast cells are treated with the test substances and the induction of β-galactosidase is measured. A positive response is indicated by a dose-related increase in the induction of β-galactosidase. For an assessment of relative potency, the induction may be compared to the results from a reference estrogen. For antagonism studies, the cells are exposed simultaneously to the test substance and the reference estrogen; control cells are exposed to the reference estrogen only. The difference in β-galactosidase activity in the presence and absence of the test substance is used as a measure of estrogen antagonism.

2.4 *In Vitro* Mammalian Cell ER TA Assays Using Growth as an Endpoint

Although most *in vitro* ER TA studies have usually measured transcription of a reporter gene to assess ER-dependent transcriptional activation, investigators have also used assays that measure cell proliferation in a cell line in response to exposure to estrogens. In these cell lines, stimulation of cell growth is a consequence of the activation of relevant genes by estrogenic substances, presumably through a receptor-binding and activation mechanism. Although cell proliferation is not a direct measure of TA *per se*, it is a measure of the cellular consequence of the interaction of the estrogen-ligand complex with ERE. The process is relevant also to the understanding of the biochemical effects of estrogens on reproductive tissues, because one of the

outcomes of estrogen stimulation *in vivo* is tissue growth. One study considered for this BRD investigated the proliferative potential of four different MCF-7 cell lines, (BUS, ATCC, BB, BB 104), and found that the BUS cell line was the most responsive to estrogens (Villalobos et al., 1995). Besides various MCF-7 cell lines, the T47D, BG-1, ELT3, and ZR-75 cell lines have been used to measure cell proliferation. These four cell lines also express an endogenous ER (**Table 2-3**).

The following section provides a generic example of how ER TA assays with mammalian cells containing endogenous ER are typically conducted when cell proliferation is used as the endpoint.

Mammalian cells containing an endogenous hER are seeded in 12-well plates at a concentration of 20,000-50,000 cells per well, in an appropriate medium (e.g., Delbecco's modified Eagle's medium) containing 5% fetal bovine serum (FBS), and grown for 24 hours at 37°C to allow the cells to attach to the plastic surface (Soto et al., 1995). The number of cells seeded can vary according to the specific protocol and cell type used. The medium is removed and replaced with fresh medium lacking phenol red and containing FBS that has been charcoal stripped to remove contaminating hormones. Various concentrations of the test substance are added and the cells are allowed to grow for six days at 37°C. A number of procedures can be used to quantitate total cell growth. These include, for example, counting cell number, or by staining and then lysing the cells and measuring a colored dye absorbed by the cells. In one dye method, the cells are fixed and stained by treating the plates with cold trichloroacetic acid at 4°C for 30 minutes, washing the cells with tap water, and allowing them to dry. The fixed cells are stained for 10 minutes with 0.4% sulforhodamine dissolved in 1% acetic acid, washed with 1% acetic acid, and air dried. The bound dye is solubilized with 10 mM Tris base, pH 10.5, in a shaker and aliquots of each well placed in a microtiter plate and read at 492 nm (Skehan et al., 1990).

The parameter of growth generally calculated is relative proliferative effect (RPE). This parameter is calculated as the ratio (x100) between the concentration of the reference estrogen and the test substance that was required to elicit a maximal cell yield after seeding an appropriate number of cells/well. Alternatively, the relative proliferative potency (RPP) is calculated. This

is the ratio of the minimal concentration of the reference estrogen needed for maximal cell yield to the minimal concentration of test substance needed to obtain a similar effect.

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